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Reassessment of the toxin profile of *Cylindrospermopsis raciborskii* T3 and function of putative sulfotransferases in synthesis of sulfated and sulfonated PSP toxins

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ABSTRACT

The toxigenic freshwater cyanobacterium *Cylindrospermopsis raciborskii* T3 has been used as a model to study and elucidate the biosynthetic pathway of tetrahydropurine neurotoxins associated with paralytic shellfish poisoning (PSP). There are nevertheless several inconsistencies and contradictions in the toxin profile of this strain as published by different research groups, and claimed to include carbamoyl (STX, NEO, GTX2/3), decarbamoyl (dcSTX), and N-sulfocarbamoyl (C1/2, B1) derivatives. Our analysis of the complete genome of another PSP toxin-producing cyanobacterium, *Raphidiopsis brookii* D9, which is closely related to *C. raciborskii* T3, resolved many issues regarding the correlation between biosynthetic pathways, corresponding genes and the T3 toxin profile. The putative *sxt* gene cluster in *R. brookii* D9 has a high synteny with the T3 *sxt* cluster, with 100% nucleotide identity among the shared genes. We also compared the PSP toxin profile of the strains by liquid chromatography coupled to mass spectrometry (LC-MS/MS). In contrast to published reports, our reassessment of the PSP toxin profile of T3 confirmed production of only STX, NEO and dcNEO. We gained significant insights via correlation between specific *sxt* genes and their role in PSP toxin synthesis in both D9 and T3 strains. In particular, analysis of sulfotransferase functions for SxtN (N-sulfotransferase) and SxtSUL (O-sulfotransferase) enzymes allowed us to propose an extension of the PSP toxin biosynthetic pathway from STX to the production of the derivatives GTX2/3, C1/2 and B1. This is a significantly revised view of the genetic mechanisms underlying synthesis of sulfated and sulfonated STX analogues in toxigenic cyanobacteria.

1. Introduction

Cylindrospermopsis raciborskii was described as a planktonic cosmopolitan filamentous cyanobacterium (Order Nostocales) from freshwater, able to fix nitrogen in terminal heterocysts (Woloszynska, 1912). *C. raciborskii* has become one of the most notorious blue-green algal species (Padisák, 1997) because of its toxicity and tendency to form dense blooms that interfere in multiple ways with water use. The species comprises strains that can produce either the hepatotoxin cylindrospermopsin (CYN), a potent protein synthesis inhibitor, or the neurotoxins saxitoxin and its analogues. The latter toxins are associated with paralytic shellfish poisoning (PSP), which can cause illness and even death of humans after consumption of seafood contaminated with these toxins, and are also responsible

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for deleterious effects on organisms in aquatic ecosystems (Ressom et al., 1994; Zingone and Enevoldsen, 2000). Nevertheless, some strains of *C. raciborskii* do not produce any known toxins.

The first report of PSP toxin production in *C. raciborskii* characterized the toxin profile of three strains T1, T2 and T3 (Lagos et al., 1999). Strains T2 and T3 were isolated in 1996 from the same location in Brazil, a branch of Billings water reservoir called Taquacetuba. The toxin profiles of cultured isolates were determined by liquid chromatography with fluorescence detection (LC-FD) after post-column derivatization. Confirmation of toxin identity was provided by liquid chromatography with detection by electro-spray ionization mass spectrometry (LC-ESI-MS). Lagos et al. (1999) mentioned that strains T2 and T3 had identical toxin profiles, comprising saxitoxin (STX), and the epimers gonyautoxin 2 and gonyautoxin 3 (GTX2/3) in a 1:9 ratio, but showed only the LC-chromatograms of strains T1 and T2. Curiously, despite the lack of definitive chromatographic evidence of the toxin profile of T3, this strain was subsequently selected by different researchers to investigate the biosynthetic pathway for PSP toxins. The first published chromatogram for *C. raciborskii* T3 (Pomati et al., 2003) was based upon a pre-column oxidation method followed by liquid chromatographic separation of the oxidation products (Lawrence et al., 1996). Furthermore, although Pomati et al. (2003) used a mixture of STX and GTX2/3 as a calibration standard and claimed to find only STX in T3, they noted that the strain also produces the low potency N-sulfocarbamoyl derivatives C1/2, by referencing the work of Lagos et al. (1999). This was a misquotation of the T3 toxin profile as described by Lagos et al. (1999), who reported only the presence of STX and GTX2/3. This confusion was later compounded by Pomati et al. (2004), who considered the "STX and C1/2" profile of T3 as the basis for experimentation and hypothesis of a possible new pathway for synthesis of C1/2 analogues, thereby modifying the biosynthetic pathways proposed by Shimizu (1996) and Sako et al. (2001).

The sequence of the PSP toxin biosynthesis gene cluster, known as the *sxt* gene cluster, in strain T3 was recently published (Kellmann et al., 2008). Based upon their LC–MS analysis, Kellmann et al. (2008) described yet another toxin profile for T3: STX, neosaxitoxin (NEO), decarbamoyl saxitoxin (dcSTX) and the N-sulfocarbamoyl toxin B1 (GTX5). These authors did not refer to synthesis of C1/2 analogues, but confusingly in the discussion they mentioned that both *Anabaena circinalis* AWQC131C and *C. raciborskii* T3 produce N-sulfonated and O-sulfated analogues of STX (B1, C2/C3, dcGTX3/dcGTX4). Kellmann et al. (2008) analyzed the structure of the gene cluster and the possible function of each open reading frame (ORF) based on the toxin profile they reported.

Obviously the interpretation of existing reports on the toxin profile and molecular genetic evidence for the biosynthetic pathway to STX and analogues in strain T3 has been complicated if not confounded by these discrepancies. The inconsistencies could conceivably have arisen via a combination of cross-contamination, miss-identification, inappropriate application or interpretation of analytical methodologies, and/or errors in citation of the literature. Furthermore, the Brazilian group responsible for the distribution of the original strain T3 did not specify the toxin profile of this strain, and only expressed the sum of all peak areas of toxins as concentration equivalents of STX (Ferrão Filho et al., 2008).

We recently sequenced the genome of *Raphidiopsis brookii* D9 (Stucken et al., 2010), a PSP toxin-producing cyanobacterium closely related to and formerly assigned to *C. raciborskii* (Stucken et al., 2009) and later to *Raphidiopsis* (Plominsky et al., 2009). The toxin profile for strain D9, based upon detailed LC–MS/MS analysis, comprises STX, dcSTX, GTX2/3 and dcGTX2/3. We identified a gene cluster for PSP toxin synthesis that is highly similar to that published for T3, but in D9 the cluster is not flanked at one end by the proposed regulatory genes *sxtY*, *sxtZ* and *ompR* and lacks four open reading frames (ORFs) related to PSP toxin production in T3 (*sxtN*, *sxtX*, *sxtW* and *sxtV*) (Stucken et al., 2010). Considering the putative differences in toxin profiles between T3 and D9, these four ORFs are of interest to evaluate and correlate the toxin profile in *C. raciborskii* T3.

The primary aim of this study was to clarify the toxin profile of *C. raciborskii* T3, a reference strain in the study of PSP toxin biosynthesis, and to assess the coherence between the PSP toxin profile and the gene content, with reference to other cyanobacterial strains. We provide further evidence on the role of sulfotransferase-like genes in the synthesis of sulfated and sulfonated analogues in cyanobacteria.

2. Materials and methods

2.1. Cyanobacterial cultures and growth conditions

The non-axenic *C. raciborskii* T3 strain was kindly provided by Sandra Azevedo (Universidade Federal do Rio de Janeiro, Brazil). *C. raciborskii* CS-505, 506 and 511 were isolated from Australia and obtained from the CSIRO collection, Hobart, Tasmania. *R. brookii* D9 was isolated by re-cloning from the multiclonal isolate SPC338 collected in 1996 from the Billings freshwater reservoir near Sao Paulo (Brazil) (Castro et al., 2004). Cyanobacteria were cultured in MLA medium according to Castro et al. (2004), at 25–28 °C under fluorescent light at a photon flux density of 40 μmol m^{-2} s^{-1} on a light/dark photocycle of 12:12 h without aeration.

In order to demonstrate the morphological identity of *C. raciborskii* T3, as directly received from S. Azevedo, who is responsible for maintenance and distribution of the original stain, the isolate was examined and compared with our previous results. The morphological identification was assessed by light microscopy with a Nikon ECLIPSE TS100 inverted microscopes at 1000× magnification with oil immersion.

2.2. Genomic DNA isolation, amplification and sequencing

The DNA was extracted with the CTAB method described by Wilson (1990). For PCR amplification of 16S rDNA, the general primers CYA106a (forward) and 1492 (reverse) were used as described by Nübel et al. (1997) and Lane et al. (1985), respectively. Amplification of the putative *sxt* gene cluster was carried out with *sxtA*, *sxtN*, *sxtX*, *sxtW*, *sxtV*, *sxtD*-context, and *sxtO*-context primers (forward